Immature Dense Granules in Platelets From Mice With Platelet Storage Pool Disease


Mepacrine uptake into platelets and bone marrow megakaryocytes was analyzed to further characterize the dense granule defects in a group of seven mouse pigment mutants that have characteristics of platelet storage pool disease (SPD). In contrast to our previous studies using electron microscopy, this method revealed that all mutants had normal numbers of dense granules. However, total mepacrine uptake in all mutant platelets was significantly diminished to less than 50% of normal uptake. Also, the flashing phenomenon observed when normal dense granules are irradiated with ultraviolet light was either greatly diminished or absent when platelets of individual mutants were similarly irradiated. Therefore the principal defect in the mutant platelets is an inability to accumulate dense granule contents rather than an absence of the granules. Mepacrine uptake into megakaryocytes was indistinguishable in normal and mutant mice. This indicates the mutant dense granule defects appear either very late in megakaryocyte development or early in platelet formation in correlation with development of the mature dense granule. By standard transmission electron microscopy we have not been able to detect gross structural or subcellular abnormalities in either platelets or megakaryocytes of mutant mice. It appears all seven mutants produce immature or functionally abnormal dense granules.

METHODS

Animals. Normal C57BL/6J and congenic or coisogenic homozygous mutants—beige (bg/bg), pearl (pe/pe), pallid (pa/pa), light ear (le/le), pale ear (pe/pe), maroon (ru-ru/ru-ru), and ruby (ru-ru)—were either obtained from the Jackson Laboratory, Bar Harbor, ME, or were bred in our animal facilities. Animals approximately 2 to 4 months of age and of both sexes were used. The properties of these pigment mutants, which exhibit symptoms of platelet storage pool diseases, are reviewed by Novak et al., and their history is reviewed by McGarry et al. Isolation of platelets and megakaryocytes. Animals were killed by anoxia with CO₂. Blood was collected by heart puncture into a 22-gauge needle attached to a 1-mL syringe containing 0.1 mL 3.8% sodium citrate. Platelet-rich plasma was isolated by centrifugation ten minutes at 150 g. Megakaryocytes were flushed from each femur with 0.75 mL "mega" medium containing 1 mmol/L adenosine, 2 mmol/L theophylline, 18.7 mmol/L Hepes buffer, 100 mmol/L glycine, 5 mmol/L EDTA, and 5% fetal calf serum in calcium and magne-

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Submitted June 30, 1986; accepted November 21, 1986.

Supported in part by NIH Grant No. HL 31098.

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sium-free Hanks' balanced salt solution, pH 7.0, containing 40 U/mL of DNase.\textsuperscript{27} The bone marrow cell suspension (3 to $5 \times 10^{8}$ cells/mL) was filtered through a wire 200 mesh filter, then partially purified first by Percoll gradients followed by Ficoll density gradient (2% to 5% Ficoll) centrifugations according to Rabellino et al.\textsuperscript{27} In later procedures the Percoll gradient fractionation was eliminated, since the percent purification was unchanged and yield was improved. Filtered bone marrow cell suspension (1.5 mL) was layered onto a 12.0 mL 2% to 5% Ficoll gradient and spun at 100 g for ten minutes. The pellet was resuspended in 1.0 mL mega medium, layered onto a second 6-mL 2% to 5% Ficoll gradient, and centrifuged at 100 g for ten minutes. The pellet was resuspended in 1.0 mL mega medium and used immediately. Samples were stained for acetylcholinesterase (specific for rodent megakaryocytes and precursors,\textsuperscript{27} to monitor the purification. Typically, partially purified preparations contained 10% megakaryocytes. In control experiments it was found that both small and large bone marrow cells accumulated mepacrine in granules. To selectively analyze megakaryocytes for mepacrine uptake, only cells larger than 20 μm in diameter were monitored. Analysis of partially purified megakaryocyte preparations revealed that 60% of acetylcholinesterase-positive cells had diameters larger than 20 μm, whereas 90% of acetylcholinesterase-negative cells had diameters less than 20 μm. Thus this procedure ensured that we were preferentially monitoring uptake into megakaryocytes, although it did not allow measurements on smaller (<20 μm) megakaryocytes.

**Mepacrine uptake.** Platelet-rich plasma containing $5 \times 10^{-3}$ mol/L mepacrine was incubated for 30 minutes at 37°C. Platelets were harvested by centrifugation at 1,000 g for ten minutes, washed twice with Tyrode's calcium-free solution, and resuspended in the above Tyrode's solution at a 3 to 5 $\times 10^{8}$ cells/mL. A drop of platelet suspension was placed on a glass slide with coverslip. After five minutes cells were analyzed in a Leitz MPV-2 fluorescent microscope with photomultiplier attachment. A mercury lamp (Osram model HBO, 200W/L2) with excitation filter at 450 to 490 nm and emission filter passing wavelengths above 510 nm was used. Platelets and megakaryocytes were analyzed under oil at 1,000 × magnification. Extraneous light was minimized by centering cells in the middle of a constricted circle formed by an iris diaphragm. The number of granules in individual cells was first counted. After a few seconds the granules began flashing. Both individual granules and granule flashes were recorded visually. Light photomicrographs of platelets were obtained in separate experiments with Kodak Tri-X Pan 400 film using the same microscope by exposing the cells to ultraviolet light for only five seconds to avoid the flashing phenomenon that occurs upon prolonged exposure to ultraviolet light.

Intensity of fluorescence (millivolts) was determined in separate platelets with a MPV-2 Leitz-Wetzlar photomultiplier together with recorder. To avoid discharge of granule contents by prolonged irradiation with UV light, the illuminated field was changed after examination of each cell. Standard curves with known concentrations of mepacrine in solution over the entire range of intensities recorded in platelets and megakaryocytes verified that the response was linear.

**Electron microscopy.** Platelets and partially purified megakaryocytes were prepared for electron microscopy according to White.\textsuperscript{27,28} Specimens for electron microscopy were initially fixed in 0.1% glutaraldehyde in White's buffer and washed in buffer before fixation in 3% glutaraldehyde in Sorensen's phosphate pH 7.3 or White's buffer. They were rinsed in phosphate buffer, postfixed in 1% Osmium tetroxide, and embedded in Epon-Araldite. Sections were cut on an LKB III ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Siemens 101 electron microscope. Samples from two normal and two of each of the mutant mice were analyzed.

## RESULTS

**Mepacrine accumulation in platelets.** Mepacrine was observed (Fig 1) to accumulate in discrete granules in normal and mutant platelets. Platelet cytoplasm contained only diffuse weak fluorescence. Photographs of platelets of three mutants—beige, pallid, and pale ear—are presented, but qualitatively similar results were obtained in all seven mutants of this study. Granules in normal platelets were more intense (see below) than in mutants, indicating an increased capacity to accumulate mepacrine.

When the number of platelet granules that accumulate mepacrine was enumerated (Table 1), there was in fact no significant difference between normal platelets and platelets from each of the seven mutants. All had five to six dense granules on average per platelet. One possible explanation of this result was that lysosomes were being counted as dense granules in mutant platelets. Mepacrine is an amine that is classified as a weak base. Weak bases are known to accumulate in lysosomes in several cell types.\textsuperscript{26,27} To test whether mepacrine was taken up into lysosomes in addition to dense granules, we took advantage of the fact that both normal and beige platelets take up and secrete serotonin from dense granules after thrombin stimulation, while only beige platelets are selectively inhibited in lysosomal enzyme secretion.\textsuperscript{1} Thus if mepacrine were taken up primarily into dense granules, mepacrine-containing granules would be equally and efficiently secreted on thrombin stimulation from both normal and beige platelets. If lysosomes were involved, normal platelets would secrete more mepacrine-containing granules than beige platelets. When thrombin-treated platelets were examined by light microscopy, both normal and beige platelets secreted all mepacrine-containing granules, indicating that in mouse platelets mepacrine accumulates primarily in dense granules. Skaer et al.\textsuperscript{18} have likewise presented evidence that mepacrine stains the dense bodies of platelets, not platelet lysosomes.

When an individual platelet was illuminated with ultraviolet (UV) light, discrete mepacrine-containing granules were observed. However, within a few seconds the granules began to flash and subsequently to fade. The flashing phenomenon, while incompletely understood, is thought to be a qualitative measure of the physical/chemical environment within the dense granule.\textsuperscript{17} Illumination with UV light probably causes release of mepacrine into cytosol where it is less quenched, thus resulting in “flashing.” It is apparent (Table 1) that there are marked differences in the flashes/platelet in normal and mutant mice. In normal mice the flashes per platelet are identical to the number of dense granules per platelet, indicating each dense granule flashes. In contrast, platelets of beige, pearl, and pallid mice exhibit little or no flashing (3% to 6% normal values). Higher rates of flashing were apparent in the light ear and pale ear mutants (43% and 37%, respectively, of normal) and the ruby-eye and maroon mutants (25% and 17% of normal). Thus in each of the mutants the number of flashes/granule is reduced, although the number of mepacrine-containing granules is normal.

Mepacrine accumulation during a 30-minute incubation was measured as the relative fluorescence intensity/platelet.
Platelets from all mutants accumulated less mepacrine than normal platelets. Of interest is the fact that the beige, pearl, and pallid mutants that were most abnormal in regard to the flashing phenomenon also accumulated the least mepacrine, only 19%, 16%, and 17%, respectively, of normal platelets. In the remaining four mutants, mepacrine accumulation was between 27% and 50% of normal accumulation.

**Mepacrine uptake into megakaryocytes.** Mepacrine uptake into partially purified large (>20 μm) megakaryocytes of bone marrow of normal and mutant mice was quantitated by fluorescence (Table 2). No significant difference in intensity was noted between normal and mutant cells. The variation in intensity within normal or mutant megakaryocytes was large. Plots of intensity vs size (not shown) showed that this variation in mepacrine uptake was not correlated with cell diameter. It was also noted that the intensity of all megakaryocytes per unit cell volume was much less than that of platelets (compare Tables 1 and 2) given the fact that megakaryocytes may give rise to about 4,000 to 8,000 platelets.

Although individual mepacrine-containing organelles were clearly visible in normal and mutant megakaryocytes, we did not attempt to count them because it was not possible to visualize all organelles in one focal plane in the relatively thick megakaryocytes. A significant finding was that flashing of mepacrine-labeled granules was observed in a small number (~1%) of normal megakaryocytes but was never observed in megakaryocytes from any of the seven mutants.

**Electron microscopic analysis of normal and mutant platelets and megakaryocytes.** Standard transmission electron microscopy of osmium-fixed thin sections revealed no gross structural abnormalities in mutant platelets (Figs 2

<table>
<thead>
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<th>Table 1. Mepacrine Uptake in Platelet Granules</th>
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<tr>
<td>Granules/Platelet</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Normal C57BL/6J</td>
</tr>
<tr>
<td>Beige</td>
</tr>
<tr>
<td>Pearl</td>
</tr>
<tr>
<td>Pallid</td>
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<td>Light ear</td>
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<td>Ruby-eye</td>
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Characteristics of dense granules of normal and mutant platelets as revealed by mepacrine uptake determined by fluorescence microscopy. Values represent the mean ± SEM of 20 to 25 platelets in each case. Relative intensity/platelet was determined prior to commencement of the flashing phenomenon. Values in parentheses are the % intensity compared to the intensity of C57BL/6J platelets, normalized to 100%.

*P < 0.001.
†P = 0.01.
Table 2. Mepacrine Uptake Into Megakaryocytes

<table>
<thead>
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<th>Relative Intensity (mV/cell)</th>
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<tr>
<td>Normal C57BL/6J</td>
<td>6900 ± 740 (28)</td>
</tr>
<tr>
<td>Beige</td>
<td>5320 ± 840 (36)</td>
</tr>
<tr>
<td>Pearl</td>
<td>4960 ± 540 (65)</td>
</tr>
<tr>
<td>Pallid</td>
<td>5980 ± 620 (30)</td>
</tr>
<tr>
<td>Light ear</td>
<td>4340 ± 660 (40)</td>
</tr>
<tr>
<td>Pale ear</td>
<td>6300 ± 720 (30)</td>
</tr>
<tr>
<td>Ruby-eye</td>
<td>6460 ± 720 (50)</td>
</tr>
<tr>
<td>Maroon</td>
<td>6360 ± 880 (40)</td>
</tr>
</tbody>
</table>

Quantitative fluorescence values represent the mean ± SEM of the indicated number (in parenthesis) of megakaryocytes.

DISCUSSION

A major conclusion of this study is that all seven mouse pigment mutants, previously characterized as animal models of platelet storage pool disease and as having very few dense granules by electron microscopic analysis of unfixed, whole-mounted platelets, actually contain normal numbers of immature dense granules. The mutant platelet granules, however, are qualitatively abnormal. They accumulate much less mepacrine than normal granules and exhibit fewer flashes. In both of these properties, the mutants are divisible into two major groups. The beige, pearl, and pallid platelets have a near absence of flashes and less than 20% normal mepacrine accumulation, while light ear, pale ear, ruby-eye, and maroon platelets flash at 17% to 44% the normal frequency and accumulate 27% to 50% the normal mepacrine concentrations. In previous experiments, it was likewise found that the mutants were separable into the same two groups when concentrations of platelet-dense granule contents such as ATP and serotonin were examined. A likely explanation for the correlation between lowered mepacrine accumulation and decreased platelet-dense granule contents is that mepacrine has been shown to bind with high affinity to dense granule components such as ATP.27 The lowered accumulation of mepacrine in mutant platelets is in agreement with the previously determined lowered rate of accumulation of another dense granule component, serotonin, in the pearl, pallid, light ear, and pale ear mutants. Taken together the data suggest platelets from all seven mutants have normal numbers of immature platelet-dense granules that are unable to retain normal quantities of dense granule components.

The results of these experiments can be compared to studies on various human and animal platelet storage pool diseases. In humans the two most studied forms of storage pool disease are Chédiak-Higashi (CHS) syndrome3 and Hermansky-Pudlak syndrome (HPS)4,5 Several studies of CHS platelets have demonstrated reduced levels of dense granule contents such as serotonin, ADP, and ATP. Most but not all morphological analyses of human CHS platelets have revealed decreased numbers of dense bodies. In platelets from animal models of CHS storage pool disease,1,2,4,19,32,33 symptoms similar to that of human CHS occur. Morphological studies by transmission electron microscopy using White's method24 and the uranaffin fixation method35 revealed a marked reduction of dense granules per platelet in cattle,24 mink,24 cat,24 and beige mouse models of CHS. However, when mepacrine labeling was used, only a small (35%) reduction of dense granules was observed in the beige mouse model.36 In addition, similar to our results, the relative intensity of mepacrine accumulation...
was one-half normal, and flashing of beige platelet granules was virtually absent.\textsuperscript{34}

Several groups have studied platelet dense granules in HPS. By morphological studies using White's method\textsuperscript{27} and the uranaffin method,\textsuperscript{34} dense granule numbers were greatly reduced in platelets from HPS. By mepacrine labeling using platelets from the same patients as the uranaffin method,\textsuperscript{34} 50\% to 70\% of normal platelet-dense granules were found, and fluorescence intensity was 15\% to 42\% of normal. Flashing was measured in one patient and found to be 10\% of normal levels.\textsuperscript{34} Mepacrine studies with platelets from the fawn-haired rat, an animal model for HPS, revealed 86\% the normal dense granule number, 81\% normal intensity, and 23\% normal flashing. These results are similar to those obtained for the pale ear, light ear, ruby-eye, and maroon mutants.

We believe it is likely the studies\textsuperscript{29,34,35,37,38} reporting large decreases in numbers of platelet-dense granules in CHS and HPS reflect limitations of the methods used. Two laboratories\textsuperscript{34,37} used White's method\textsuperscript{24,25} to analyze thin sections, others\textsuperscript{34,38} the uranaffin method,\textsuperscript{35} and still another\textsuperscript{29} the whole-mount electron microscopic method.\textsuperscript{39} These techniques depend upon having normal or near normal concentrations of dense-granule contents for identification in the electron microscope. This is true for the whole-mount method, which uses no fixatives and relies upon the natural density of the granule, and for the uranaffin method, which depends upon reaction with ATP in the granule. In White's method external calcium is included in the fixation medium to enhance dense granule contrast, but it is uncertain if this procedure adequately labels all dense granules in SPD patients. It is commonly found that other externally added components, such as serotonin and mepacrine, accumulate in dense granules of SPD patients to a much lower degree than in dense granules of normal patients. On the other hand, the mepacrine-labeling method utilized to study SPD platelets in this study and others\textsuperscript{36,38,40,41} is a sensitive fluorometric method that is easily capable of revealing dense granules, even if only 15\% to 20\% the normal concentration is taken up into dense granules. We have analyzed the same normal and mutant mouse platelets by the mepacrine (this report) and whole-mount method.\textsuperscript{1} It was found that the latter method revealed greatly reduced numbers of dense granules, while the mepacrine technique indicated normal dense granule number. Similarly, Richards et al\textsuperscript{35} and Lorez and Da Prada\textsuperscript{38} analyzed platelets of beige mice by the mepacrine and uranaffin methods and found no dense granules by the latter method but near normal numbers by the former. Rendu et al\textsuperscript{42} analyzed normal and CHS platelets by the mepacrine technique and by White's method. While both methods revealed decreased contents of dense granules in CHS patients, the decrease was much larger with White's method. Thus it appears likely that the whole-mount, uranaffin, and White's methods are well suited for revealing "full" granules, while the mepacrine-labeling method detects both "full" and "empty" granules. We believe, therefore, that taken together, the available data suggest that while there is a partial reduction of platelet-dense granules in certain cases,\textsuperscript{32,41} the platelet-dense granule defect in SPD is due to major qualitative abnormalities in dense granules that impair their ability to accumulate normal contents rather than to an absence of dense granules per se.

Total mepacrine uptake in our studies into megakaryocytes, in contrast to platelets, was relatively low and also was not significantly different in normal and mutant mice. Thus the mutant dense-granule defects (ie, absence of dense granule contents) appear either very late in megakaryocyte development or early in platelet formation. The finding that a few (~1\%) normal megakaryocytes flashed while no flashing was detectable in any mutant megakaryocytes suggests that only a small population of megakaryocytes, possibly a very late stage in maturation, contains mature dense granules. Since flashing was never seen in the mutant cells, the mutant defects likely are already present in megakaryocytes. The absence of flashing of dense granules of mutants illustrates that mutant dense granules express their immature phenotype when formed.

The low mepacrine accumulation in megakaryocytes is consistent with what is known of the timing of the accumulation of dense granule contents in normal platelets. For example, Mishory and Danon\textsuperscript{42} found that newly formed platelets entering the bloodstream have practically no osmophilic organelles and that the number and size of these organelles increases with the age of the platelets.

The fact that mepacrine uptake is equal in normal and mutant megakaryocytes is consistent with the idea that the dense granule defect in all mutants is not in production of dense granules, since immature dense granules are present in mutant megakaryocytes. Rather, the defect seems to reside in the ability of dense granules to accumulate normal contents. Previous experiments utilizing bone marrow transplantation have shown that factors extrinsic to bone marrow precursor cells are not defective.\textsuperscript{15,16} Also, initial rates of accumulation of one dense granule component, serotonin, are normal,\textsuperscript{14} suggesting a normal transport mechanism. The defects in mutant platelets, therefore, are more likely to lie in mechanisms for retention of dense-granule components.

It is possible that some other substance(s) is present in mutant dense granules that prevents accumulation of dense-granule components and inhibits flashing. This is unlikely, at least in regard to primary gene products, since it is expected that a recessive mutation would result in a deficiency, rather than an increase, in the gene product. It remains possible that accumulation of such hypothetical substances could occur in mutant granules as a secondary effect of the mutations.

One uncertainty in interpretation of our megakaryocyte data is that, in comparison to platelets, there is less independent evidence that mepacrine uptake is principally into dense granules. The fact, however, that megakaryocytes incorporate labeled serotonin\textsuperscript{49} is evidence that they are capable of concentrating normal dense-granule components. Also, Hourdille et al\textsuperscript{44} considered their finding of lowered mepacrine accumulation in megakaryocytes of a patient with a dense-granule defect as evidence that the mepacrine is incorporated mainly into dense granules.

Relatively few studies have been performed on dense granules of megakaryocytes of human storage pool disease. Hourdille et al\textsuperscript{44} found, in contrast to our results with mutant mice, that 74\% of megakaryocytes of a patient with HPS showed no labeling with mepacrine and concluded,
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therefore, that the platelet anomaly is secondary to a megakaryocyte anomaly. Richards and Da Prada\textsuperscript{35} found no positive uranaffin reaction in megakaryocytes of HPS patients or beige mice or fawn-hooded rats. However, this same method of detection also revealed\textsuperscript{44} no positive reaction in platelets of the above mutant animals while we and Lorez et al\textsuperscript{38} have readily detected dense granules in platelets of these mutants using the mepacrine-labeling technique. Therefore, as previously discussed, the mepacrine method may be a more sensitive method of detecting the relatively immature dense granules of megakaryocytes. Further studies on megakaryocytes of SPD patients are needed for an adequate comparison to our results using the various mouse mutants.

These results show that the normal accumulation of dense granule contents is under multigenic control with at least seven genes involved. The molecular bases for the organelle defects in the mouse mutants are still unknown. A speculative explanation of our results is that the mutants have defects in the membrane of dense granules. Probably such defects are also present in lysosomes and melanosomes,\textsuperscript{39} emphasizing the relatedness of the three organelles. Our current focus is on identifying the primary gene products involved in the mouse models of SPD.

ACKNOWLEDGMENT

We thank Cheryl Mrowczynski for excellent secretarial aid and Al Cairo for aid in production of the photomicrographs.

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